The Plant Cell, Vol. 15, 965–980, April 2003, www.plantcell.org © 2003 American Society of Plant Biologists

The Pleiotropic Role of the 26S Proteasome Subunit RPN10 in Arabidopsis Growth and Development Supports a Substrate-Specific Function in Abscisic Acid Signaling

Jan Smalle,[a] Jasmina Kurepa,[a] Peizhen Yang,[a] Thomas J. Emborg,[a] Elena Babiychuk,[b] Sergei Kushnir,[b] and Richard D. Vierstra[a,1]

[a] Department of Horticulture and Cellular and Molecular Biology Program, University of Wisconsin–Madison, Madison, Wisconsin 53706

[b] Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

The 26S proteasome is an essential protease complex responsible for removing most short-lived intracellular proteins, especially those modified with polyubiquitin chains. We show here that an Arabidopsis mutant expressing an altered RPN10 subunit exhibited a pleiotropic phenotype consistent with specific changes in 26S proteasome function. *rpn10-1* plants displayed reduced seed germination, growth rate, stamen number, genetic transmission through the male gamete, and hormone-induced cell division, which can be explained partially by a constitutive downregulation of the key cell cycle gene *CDKA;1*. *rpn10-1* also was more sensitive to abscisic acid (ABA), salt, and sucrose stress and to DNA-damaging agents and had decreased sensitivity to cytokinin and auxin. Most of the phenotypes can be explained by a hypersensitivity to ABA, which is reflected at the molecular level by the selective stabilization of the short-lived ABA-signaling protein ABI5. Collectively, these results indicate that RPN10 affects a number of regulatory processes in Arabidopsis likely by directing specific proteins to the 26S proteasome for degradation. A particularly important role may be in regulating the responses to signals promulgated by ABA.

INTRODUCTION

The ubiquitin/26S proteasome pathway has been implicated in diverse aspects of eukaryotic cell regulation through its ability to rapidly remove intracellular proteins (Hershko and Ciechanover, 1998; Callis and Vierstra, 2000). In this pathway, proteins destined for degradation first become modified by the covalent attachment of polymeric ubiquitin chains. These chains are assembled on one or more Lys residues within the target via an ATP-dependent reaction cascade involving the sequential action of activating (E1), conjugating (E2), and ligating (E3) enzyme families (Hershko and Ciechanover, 1998; Gagne et al., 2002). The resulting polyubiquitinated proteins then are recognized by the 26S proteasome and degraded, with the concomitant release of the ubiquitin moieties for reuse. Through this conjugation/degradation cycle, the ubiquitin/26S proteasome pathway controls numerous physiological and developmental events by selectively removing key cell regulators (Hershko and Ciechanover, 1998). Although few of the targets are known in plants, genetic analyses have implicated the pathway in hormone regulation, embryogenesis, photomorphogenesis, circadian rhythms, floral homeosis, stress responses, senescence, and pathogen defense (Callis and Vierstra, 2000; Hellmann and Estelle, 2002).

[1] To whom correspondence should be addressed. E-mail vierstra@facstaff.wisc.edu; fax 608-262-4743.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.009217.

The 26S proteasome is an ATP-dependent, self-compartmentalized protease (Voges et al., 1999; Glickman, 2000). Although most work on this 2-MD complex is derived from yeast and animals, evidence is accumulating that the higher plant version is similar in structure and function (Fu et al., 1998a, 1999a, 1999b; Shibahara et al., 2002). The 26S proteasome can be divided further into two particles, the 20S core protease (CP) and the 19S regulatory particle (RP). The CP is a broad-spectrum ATP- and ubiquitin-independent peptidase created by the assembly of four stacked heptameric rings of related α- and β-subunits in an $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ configuration. The protease active sites within β1, β2, and β5 polypeptides are sequestered in a central chamber. Access to this chamber is restricted by a narrow gated channel created by the α-subunit rings that allows only unfolded proteins to enter (Glickman, 2000).

The RP binds to each end of the CP and confers both ATP dependence and substrate specificity to the holoenzyme, especially with respect to those substrates bearing the polyubiquitin tag (Voges et al., 1999; Glickman, 2000). The RP presumably helps identify appropriate substrates for breakdown, removes the attached ubiquitins, opens the α-subunit ring gate, and directs the entry of unfolded proteins into the CP lumen for degradation. Its 18 principal subunits can be divided further into two subparticles, the Lid and the Base. The Base contains three non-ATPase subunits (RPN1, RPN2, and RPN10) and a ring of six AAA-ATPase subunits (RPT1 to RPT6) that contacts the α-subunit ring and likely assists in target unfolding and transport (Voges et al., 1999; Glickman, 2000; Fu et al., 2001).

RPT5 also may play a role in the recognition of polyubiquitinated proteins (Lam et al., 2002). The Lid binds to each end of the Base and contains nine additional RPN subunits (RPN3, RPN5 to RPN9, and RPN11 to RPN13). The functions of most of the RPN subunits are unknown. All but two (RPN9 and RPN10) are essential in yeast. RPN11 has proteolytic activities capable of removing bound ubiquitins, implicating it in the release of polyubiquitin chains from substrates (Verma et al., 2002). RPN1 has been proposed to play a role in the recognition of ubiquitin-like sequences (UBLs) that may help deliver substrates to the 26S proteasome (Elsasser et al., 2002).

Several roles have been ascribed to RPN10. It was identified originally by its ability to bind polyubiquitin chains in vitro (van Nocker et al., 1996a). Because its preference for ubiquitinated proteins bearing chains with four or more ubiquitins matches that of the 26S proteasome, RPN10 initially was considered to be the main ubiquitinated protein receptor in the 26S proteasome complex (van Nocker et al., 1996a; Piotrowski et al., 1997). van Nocker et al. (1996b) subsequently showed that a yeast *Δrpn10* strain is viable but hypersensitive to amino acid analogs that create damaged proteins and is compromised in the degradation of only a subset of ubiquitin/26S proteasome targets. The *Δrpn10* phenotype indicated that RPN10 is not the sole ubiquitin receptor but plays a more substrate-specific role. The ubiquitin-interacting motif (UIM) was identified as a hydrophobic patch near the C terminus of the protein (Fu et al., 1998b; Hofmann and Falquet, 2001). Contrary to expectations, this domain is not essential for the observed functions of RPN10 in amino acid analog resistance or specific protein breakdown, indicating that these functions are conferred by other domain(s) (Fu et al., 1998b).

Another role for RPN10 may be in stabilizing the RP. Loss of RPN10 allows the yeast Lid and Base to easily dissociate in vitro, suggesting that it helps tether the two subcomplexes (Glickman et al., 1998; Fu et al., 2001). A positionally conserved aspartate (Asp-11) near the N terminus of RPN10 is critical for this association (Fu et al., 2001). It sits within an ~150–amino acid region related to the von Willebrand factor A domain (vWA) fold, which may help connect the Lid to the Base. Additional roles for RPN10 also are possible based on its ability to interact genetically or physically with other proteins, including RPN1 of the Base and RPN9 and RPN12 of the Lid (Kominami et al., 1997; Takeuchi et al., 1999; Wilkinson et al., 2000; Fu et al., 2001). Synergistic phenotypes also are apparent when deletions of RPN10 are combined with those of DSK2 and the DNA-repair protein RAD23 (Chen and Madura, 2002; Saeki et al., 2002). DSK2 and RAD23 belong to a family of proteins that contain an N-terminal UBL domain and a C-terminal ubiquitin-associated domain that can bind ubiquitin. Based on the notion that the UIM and the UBL domain interact, RPN10 may work in combination with RAD23/DSK2 to deliver ubiquitinated cargo to the 26S proteasome. However, it has not been shown that RPN10 associates directly with DSK2 and RAD23; thus, it may play a more indirect role in UBL domain binding (Elsasser et al., 2002).

To further expand our understanding of RPN10 functions, we and others have begun to characterize corresponding mutants in various multicellular organisms. Like yeast *Δrpn10* strains, a *Δrpn10* disruption mutant from the moss *Physcomitrella patens* is viable, has increased levels of ubiquitin conjugates, and is hypersensitive to amino acid analogs, indicating a mild attenuation of the ubiquitin/26S proteasome pathway (Girod et al., 1999). However, the moss mutant also is developmentally arrested, being unable to make gametophores from the vegetative caulonema tissue. This arrest can be overcome partially by treating caulonema with a combination of the auxin and cytokinin hormones, suggesting that RPN10 mediates the degradation of one or more regulators of hormone action. Both the vWA domain and the UIM appear important for the phenotypic function of moss RPN10 (Girod et al., 1999). Double-stranded RNA interference of *RPN10* in *Drosophila* cultured cells increased the levels of polyubiquitinated proteins even though the derived 26S proteasomes had increased peptidase activity (Wojcik and DeMartino, 2002). Similar interference showed that *RPN10* is essential in *Trypanosome brucei* (Li and Wang, 2002). Although the mutant still could assemble 26S proteasomes without the RPN10 protein, the protozoan showed a strong growth arrest.

Here, we describe the isolation and characterization of a T-DNA mutant from the higher plant Arabidopsis expressing an aberrant form of RPN10. The *rpn10-1* line exhibited a pleiotropic phenotype, indicating that a number of processes were compromised selectively. Most strikingly, the postgerminative development of *rpn10-1* seedlings was highly sensitive to the hormone abscisic acid (ABA), salt, and sucrose stress. Many of the phenotypes appear to be caused by a hypersensitivity to ABA, reflected at the molecular level by a failure to rapidly degrade the ABA response protein ABA INSENSITIVE-5 (ABI5). Collectively, the mutant phenotypes indicate that RPN10 plays a role in delivering a specific subset of short-lived targets to the Arabidopsis 26S proteasome for degradation.

RESULTS

The T-DNA Insertion Mutant *rpn10-1*

The Arabidopsis RPN10 protein is encoded by a single gene located on chromosome 4 (TAIR locus At4g38630). To help define its functions, we searched the various mutation databases for insertional mutants in the *RPN10* locus. A T-DNA insertion of Arabidopsis ecotype C24 within the transcribed region of *RPN10* was generated fortuitously as part of an exon-trapping project that exploited a promoterless chimeric apurinic endonuclease (*ARP*)–neomycin phosphotransferase (*NPTII*) gene to identify expressed loci (Babiychuk et al., 1997). Sequence analysis of the 5′ rapid amplification of cDNA ends product from the kanamycin-resistant line SL41-45 (now designated *rpn10-1*) detected a chimeric mRNA that encoded the N-terminal part of RPN10 (residues 1 to 186) followed by the full-length ARP-NPTII protein sequence of 280 amino acids (Figure 1A). Analysis of the genomic sequence revealed that the T-DNA inserted within the fifth intron of the *rpn10-1* locus, thus creating a chimeric intron that, when spliced out, would generate a fused coding region (Figure 1A). Notably, the *rpn10-1* protein product retained the N-terminal vWA fold of RPN10 that is essential for RP stability in yeast but was missing the C-terminal half that contains

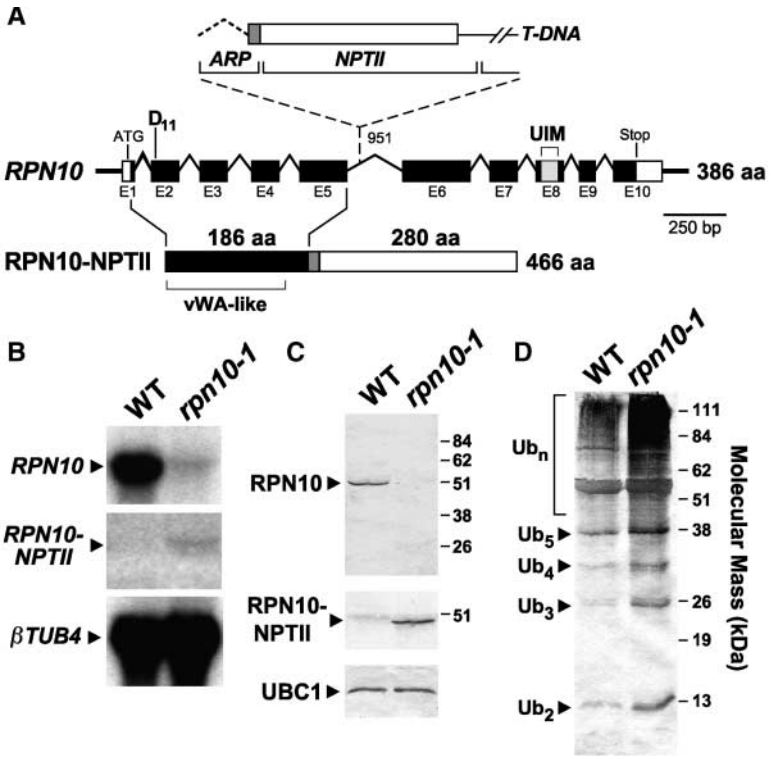

Figure 1. Molecular and Biochemical Descriptions of the Arabidopsis *rpn10-1* Mutation.

(A) Structure of the *RPN10* gene and the effect of the T-DNA insertion containing *ARP-NPTII* on the predicted protein product. Boxes and lines denote exons and introns, respectively. Upon insertion of the T-DNA into the fifth intron, the remaining intron sequence of the *RPN10* gene (bp 940 to 950) and the 5′ end of the *ARP-NPTII* gene form a chimeric intron that when spliced expresses the translational fusion product (*RPN10-NPTII*) containing the N-terminal 267 amino acids of RPN10 appended to the 280 amino acids of ARP-NPTII. aa, amino acids.

(B) Detection of *RPN10* and *RPN10-NPTII* transcripts in 2-week-old wild-type (WT) and *rpn10-1* seedlings by RNA gel blot analysis using probes for the *RPN10* and *NPTII* sequences. Equal loading of total RNA was confirmed by staining for rRNA with methylene blue (data not shown) and by reprobing the blots with β-tubulin (*βTUB4*).

(C) Detection of RPN10 and the RPN10-NPTII fusion protein. Total protein from 2-week-old wild-type and homozygous *rpn10-1* seedlings was subjected to SDS-PAGE and immunoblot analysis with anti-RPN10 and anti-NPTII antibodies. Equal protein loads were confirmed by immunoblot analysis with anti-UBC1 antibodies.

(D) Ubiquitin (Ub) conjugate levels are increased in *rpn10-1* seedlings. Equal amounts of total protein from **(C)** were subjected to SDS-PAGE and immunoblot analysis with anti-ubiquitin antibodies. The positions of free polyubiquitin chains and ubiquitinated proteins are indicated.

the UIM responsible for binding polyubiquitin chains (Fu et al., 1998b, 2001). Kanamycin resistance was tightly linked to the *rpn10-1* mutation, indicating that this line contained a single T-DNA insert (J. Smalle, unpublished data).

RNA gel blot analysis showed that the T-DNA insertion severely attenuated the accumulation of the *RPN10* mRNA. Whereas the 1.4-kp *RPN10* transcript was detected easily in wild-type plants, just a trace of the predicted 1.6-kp *RPN10-NPTII* transcript was seen in homozygous *rpn10-1* plants using a *RPN10* probe (Figure 1B). This species also was detected using a *NPTII* probe, demonstrating that the *NPTII* sequence was part of the mRNA. Accordingly, a much reduced amount of the RPN10-NPTII fusion protein accumulated in the *rpn10-1* plants. Using anti-NPTII antibodies, a protein with the expected apparent molecular mass of 50 kD was detected in total protein extracts from *rpn10-1*, indicating that the predicted full-length fusion protein was expressed (Figure 1C). The NPTII domain also was active and could confer kanamycin resistance to the seedlings. However, when anti-RPN10 antibodies were used, the level of fusion protein was nearly undetectable compared with RPN10 in the wild type (Figure 1C). (We note that wild-type RPN10 migrates anomalously during SDS-PAGE, with an apparent mass similar to that of the RPN10-NPTII fusion protein [~50 kD], even though its actual mass is 41 kD [van Nocker et al., 1996a]). Because the anti-RPN10 antibodies easily recognized the N-terminal half of the protein (Figure 2A) (Fu et al., 1998b), we concluded that this low apparent level reflects diminished synthesis and not decreased antigenicity of the RPN10-NPTII fusion protein.

Reduced levels of RPN10 have been shown in other organisms to substantially increase the levels of ubiquitinated proteins, likely by attenuating 26S proteasome activity (van Nocker

et al., 1996b; Girod et al., 1999; Wojcik and DeMartino, 2002). Arabidopsis *rpn10-1* seedlings displayed a similar effect. In addition to increased levels of high molecular mass polyubiquitinated proteins, increased levels of free polyubiquitin chains were evident (Figure 1D). This increase was not caused by reduced amounts of 26S proteasomes. In fact, immunoblot analysis indicated that the amounts of 26S proteasome subunits other than RPN10 were higher in *rpn10-1* seedlings than in wild-type seedlings (J. Smalle, unpublished data).

Loss of RPN10 significantly alters the stability of the yeast RP in vitro, allowing the Lid to dissociate from the Base under mild salt conditions (Glickman et al., 1998; Fu et al., 2001). To test for a similar effect with the Arabidopsis *rpn10-1* mutant, we partially purified 26S proteasomes from wild-type and *rpn10-1* seedlings and compared their size and stability by chromatographic methods. The CP was assayed by peptidase activity using the synthetic substrate *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin and by immunoblot analysis with antibodies against the α3-subunit PAC1 and the β6-subunit PBF1. The RP was assayed by immunoblot analysis with antibodies against RPT1 and RPN5 that detect the Base and Lid subcomplexes, respectively. Superose HR6 size-exclusion chromatography did not reveal any significant size differences between the wild-type and mutant 26S proteasome preparations (P. Yang, unpublished data). Likewise, Resource Q anion-exchange chromatography, which can detect subtle effects on RP stability (Glickman et al., 1998; Fu et al., 2001), also failed to detect differences between the two preparations. Both wild-type and mutant complexes eluted at the same salt concentration with similar profiles (Figures 2A and 2B). In both wild-type and *rpn10-1* preparations, the RP (as identified by the RPT1 and RPN5 proteins) eluted with a broader profile than the CP, with some eluting at slightly lower salt concentrations, consistent with its dissociation from the CP under these chromatographic conditions (Fu et al., 2001).

Despite the much reduced levels of the RPN10-NPTII protein in total extracts from the homozygous *rpn10-1* plants compared with wild-type plants (Figure 1C), the fusion protein was detected easily in the enriched 26S proteasome fractions using either anti-RPN10 or anti-NPTII antibody (Figure 2B). Thus, even with the NPTII appendage, the fusion protein readily incorporated into the 26S complex. This incorporation indicates that the C-terminal half of RPN10 is not necessary for RP binding and is consistent with the importance of the N terminus for

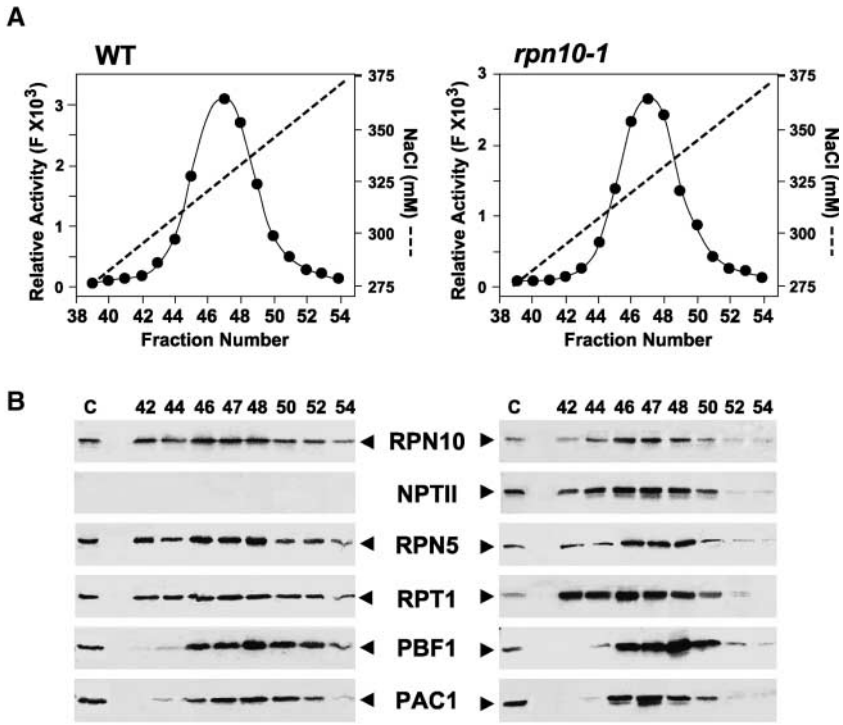

Figure 2. Association of the RPN10-NPTII Fusion Protein with the Arabidopsis 26S Proteasome.

The 26S proteasome was enriched from 10-day-old wild-type (WT) and homozygous *rpn10-1* seedlings by sequential polyethylene glycol precipitations and subjected to anion exchange fast protein liquid chromatography using a NaCl gradient for elution.

(A) Column fractions were assayed for peptidase activity of the CP using *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin as the substrate and are expressed in relative fluorescence units (F). Only the portions of the fast protein liquid chromatography elution profiles centered on the peaks of peptidase activity are shown.

(B) Column fractions were subjected to SDS-PAGE and immunoblot analysis with antibodies against NPTII, the RPN10, RPN5, and RPT1 subunits of the RP, and the PBF1 and PAC1 subunits of the CP. C, control.

RP stability (Fu et al., 1998b, 2001). The structural integrity of the 26S proteasome from the *rpn10-1* mutant strongly suggests that the resulting fusion protein does not alter the incorporation/association of the other principal subunits into the Arabidopsis 26S complex.

rpn10-1 Shows Developmental Changes throughout Its Life Cycle

Consistent with the importance of the ubiquitin/26S proteasome pathway in plants (Callis and Vierstra, 2000; Hellmann and Estelle, 2002), Arabidopsis seeds homozygous for the *rpn10-1* mutation displayed numerous developmental defects. *rpn10-1* seeds have markedly decreased germination, especially for seeds sown soon after reaching maturity. If germinated within 1 week after maturation, only 7 $\pm$ 6% of *rpn10-1* seeds germinated compared with 98 $\pm$ 2% for wild-type seeds (Figure 3A). This poor germination improved as the seeds aged, with 83 $\pm$ 5% of the *rpn10-1* seeds germinating compared with nearly 100% for wild-type seeds after 1 month of storage at room temperature.

After germination in the dark, etiolated *rpn10-1* seedlings grew similar to wild-type seedlings, except for a slight reduction in hypocotyl and root extension (Figure 3D). When grown in the light, a substantial decrease in growth rate was observed, including a slower rate of leaf initiation and expansion and a decrease in root elongation (Figure 3B and data not shown). Although the cotyledons appeared normal, the rosette leaves senesced prematurely, especially at the leaf edges (Figure 3C). This chlorosis was more pronounced in leaves that emerged early and decreased in leaves produced later in rosette development (Figures 3B and 3C). Although slower in growth, *rpn10-1* rosettes eventually reached the same size as wild-type rosettes. Later in development, the *rpn10-1* inflorescences actually grew taller, likely caused by the reduced fecundity of the mutant, delaying the arrest of its inflorescence meristems (Figure 3D) (Hensel et al., 1993). Surprisingly, most *rpn10-1* flowers had only four stamens, as opposed to the six stamens typically found in wild-type flowers (Figure 3E). Seed set from these flowers was reduced dramatically, with more than half of *rpn10-1* siliques containing no seeds and the remaining siliques containing fewer than five seeds.

Although the *rpn10-1* locus behaved as a recessive mutation, only $\sim$5% of the progeny of a self-fertilized *rpn10-1* heterozygote were homozygous for the mutation, instead of the expected 25%. This reduced frequency cannot be explained fully by the reduction in seed germination. Reciprocal crosses to the wild type using a *rpn10-1* heterozygote as the source of male and female gametes revealed that the mutant is partially male sterile. Whereas the *rpn10-1* allele was transmitted through the egg near the expected 1:1 segregation ratio (21:17 for *RPN10*/*RPN10* versus *RPN10*/*rpn10-1*), an $\sim$8:1 segregation ratio (31:4) was obtained when *rpn10-1* was transmitted through the pollen.

rpn10-1 Is Hypersensitive to DNA-Damaging Agents

The growth reduction of *rpn10-1* seedlings was more apparent under long-day photoperiods compared with short-day photoperiods (Figure 4A). In addition, the *rpn10-1* plants under long days were noticeably more chlorotic, suggesting that the mutant is photosensitive. Based on the connection of yeast RPN10 to DNA repair via its genetic interaction with RAD23 (Lambertson et al., 1999; Chen and Madura, 2002), it was possible that the photosensitivity of *rpn10-1* reflected the attenuation of a DNA repair mechanism involving a plant ortholog of RAD23 (Sturm and Lienhard, 1998). To test for a possible DNA repair defect, *rpn10-1* seedlings were exposed to increasing doses of UV-B light and the DNA cross-linking agent mitomycin C. As can be seen in Figures 4B to 4D, the *rpn10-1* mutant was more sensitive than the wild type to both treatments. A greater reduction in growth rate and increased chlorosis were evident for daily UV-B irradiations (Figure 4B and 4C). Concentrations of mitomycin C (2 μg/mL) that did not appreciably affect wild-type seedlings severely reduced the growth of *rpn10-1* seedlings, including a complete inhibition of root elongation (Figure 4D). This sensitivity was not seen for *rpn12a-1*, an Arabidopsis mutant that affects another subunit of the RP (Smalle et al., 2002), indicating that the DNA repair defect of *rpn10-1* was not caused by a general attenuation of 26S proteasome activity (J. Smalle, unpublished data).

rpn10-1 Has Decreased Cytokinin Sensitivity and Constitutive Downregulation of *CDKA;1* Expression

We previously showed that the Arabidopsis RPN12a subunit plays a role in cytokinin responses (Smalle et al., 2002). Given that RPN10 interacts with RPN12 in yeast (Kominami et al., 1997; Wilkinson et al., 2000) and that some of the *rpn10-1* phenotypes could be explained superficially by defects in cytokinin signaling (reduced growth rate and accelerated senescence), we tested for a similar decrease in cytokinin sensitivity in *rpn10-1*. As can be seen in Figure 5A, root elongation of *rpn10-1*, like that of *rpn12a-1* (Smalle et al., 2002), was substantially less inhibited by the cytokinin kinetin than that of the wild type. Whereas root growth of wild-type seedlings was suppressed strongly by as little as 0.05 μM kinetin, $>$1 μM kinetin was needed to elicit the same effect in the mutant.

The *rpn10-1* mutation also decreased the ability of cytokinins to stimulate cell division and shoot induction. As in *rpn12a-1* (Smalle et al., 2002), greening and shoot induction from hypocotyl segments by appropriate ratios of the auxin indoleacetic acid (IAA) and the cytokinin 2-isopentyladenine (2iP) were reduced markedly in the *rpn10-1* mutant (Figure 5B). However, after prolonged exposures of 3 weeks or more, which produced green calli and stimulated the emergence of shoots from the wild type and a modest induction of calli from *rpn12a-1*, cell division and callus formation were not evident from *rpn10-1* (Figure 5C). Although this response suggested that *rpn10-1* is more insensitive to cytokinins than *rpn12a-1*, the response of intact seedlings exposed continuously to kinetin implied a more complex effect. The growth of *rpn12a-1* seedlings was affected only mildly by 0.1 μM kinetin, whereas the growth of wild-type and *rpn10-1* seedlings was repressed dramatically (Figure 5D).

To further understand the roles of RPN10 in cytokinin signaling, we examined by RNA gel blot analysis the expression of a

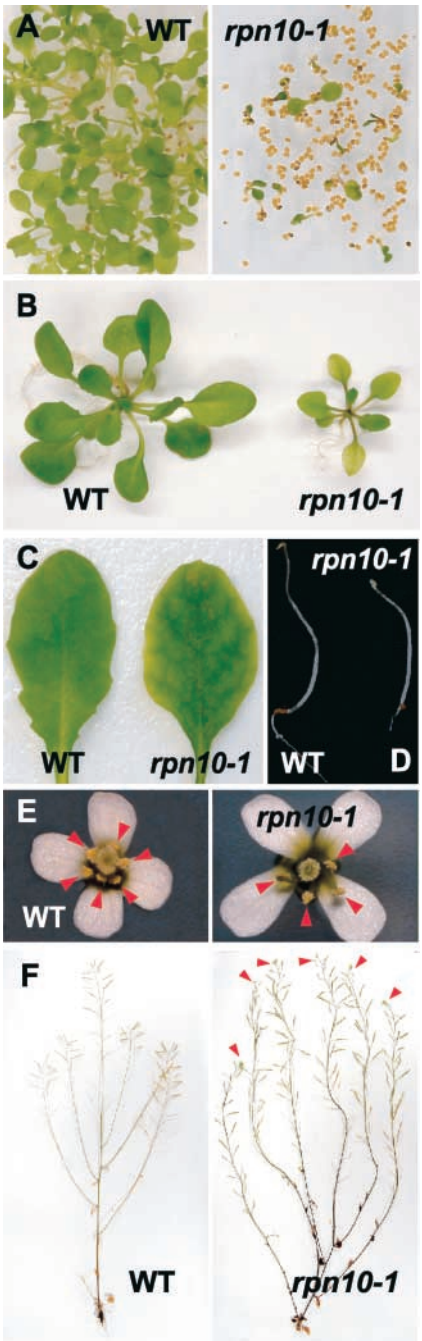

Figure 3. Developmental Changes Induced by the *rpn10-1* Mutation.

(A) Decreased germination of *rpn10-1* seeds compared with wild-type (WT) seeds.

(B) Decreased growth rate of *rpn10-1* plants during the first 4 weeks of development.

(C) Premature chlorosis of mature *rpn10-1* leaves. Shown is a rosette leaf from a 6-week-old *rpn10-1* plant exhibiting chlorosis at the edges next to a wild-type rosette leaf of comparable age.

(D) Decreased hypocotyl and root growth of etiolated *rpn10-1* seedlings.

(E) Reduced stamen number of *rpn10-1* flowers. Whereas wild-type flowers contained six stamens, the *rpn10-1* flowers typically contained four stamens (arrowheads).

battery of cytokinin-regulated genes in the *rpn10-1* background. The cytokinin early-response gene *ARR5*, which is upregulated dramatically but transiently within 15 min of cytokinin treatment, is thought to act as a feedback inhibitor of cytokinin action (D'Agostino et al., 2000; Hwang and Sheen, 2001). The basal and cytokinin-induced expression of *ARR5* is unaffected by the *rpn12a-1* mutation (Smalle et al., 2002). For the *rpn10-1* mutation, the basal *ARR5* mRNA levels were suppressed by approximately twofold compared with wild-type levels and increased by approximately twofold after a 30-min treatment with a 5-μM concentration of the cytokinin benzyladenine (BA) (Figure 5E). Expression of cytokinin late-response genes, like those that encode nitrate reductase (*NIA1*) and cyclin *D3* (*CYCD3*), is upregulated after prolonged exposure of wild-type seedlings to cytokinins (Yu et al., 1998; Riou-Khamlichi et al., 1999). Smalle et al. (2002) found that the mRNA levels for both *NIA1* and *CYCD3* are increased constitutively in *rpn12a-1*. By contrast, *rpn10-1* seedlings expressed normal levels of *NIA1* and *CYCD3* in the absence of BA but failed to upregulate their expression after a 12-h BA treatment (Figure 5F). This long exposure of *rpn10-1* to BA elicited a mild increase in the steady state level of *ARR5* mRNA (Figure 5F). Expression of the *RPN10* gene also was induced by BA (approximately threefold), similar to the cytokinin induction of *RPN12a* and other genes that encode 26S proteasome subunits (Figure 5F) (Smalle et al., 2002).

The reduced growth rate of the *rpn10-1* plants suggested that the expression and/or action of one or more cyclin-dependent kinases (CDKs) were affected. Of particular interest was the *CDKA;1* gene (previously named *CDC2a*), whose expression has been linked to plant growth rate and cell division activity (Hemerly et al., 1993; Beemster et al., 2002). Whereas the expression of *CDKA;1* is normal in *rpn12a-1* plants (Smalle et al., 2002), its expression was downregulated selectively in the *rpn10-1* mutant (Figure 5G). In contrast to the levels of two other Arabidopsis CDKs, *CDKC;1* and *CDKD1;3* (Vandepoele et al., 2002), we found that the mRNA level for *CDKA;1* was reduced at least twofold to threefold in *rpn10-1* seedlings with or without BA treatment (Figures 5E and 5G).

Rpn10-1 Roots Have Decreased Auxin Sensitivity

The *rpn10-1* mutant, like *rpn12a-1* (Smalle et al., 2002), exhibits a slight decrease in auxin sensitivity, detected by a diminished responsiveness of roots to low concentrations of 2,4-D. Auxins are known to inhibit root elongation while at the same time stimulating lateral root formation. As can be seen in Figures 6A and 6C, inhibition of root elongation and promotion of lateral roots were less pronounced in *rpn10-1* compared with the wild type on medium containing 5- to 10-nM concentrations of the

(F) Prolonged longevity of *rpn10-1* inflorescences. Arrowheads indicate active floral meristems after 8 weeks of growth under a long-day photoperiod.

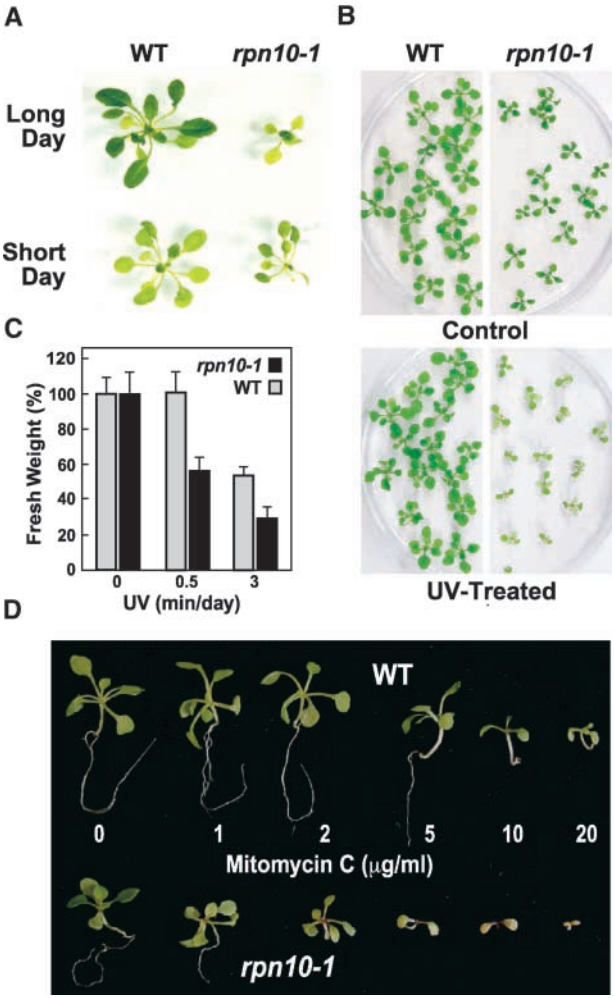

Figure 4. The *rpn10-1* Mutant Is Hypersensitive to UV-B Light and the DNA-Damaging Agent Mitomycin C.

(A) *rpn10-1* seedlings are hypersensitive to long-day versus short-day photoperiods. Wild-type (WT) and *rpn10-1* plants were grown for 3 weeks under long days (16 h of light and 8 h of dark) or for 4 weeks under short days (8 h of light and 16 h of dark).

(B) Hypersensitivity of *rpn10-1* plants to UV-B light. Seedlings were irradiated daily with 0.5 min of UV-B light. Images are of 14-day-old seedlings.

(C) Effect of daily UV-B irradiations on the accumulation of fresh weight. Seedlings were irradiated daily for 14 days with a dose of 0.5 or 3 min of UV-B light. Values shown are means from 10 seedlings (±SD).

(D) *rpn10-1* seedlings are hypersensitive to mitomycin C. Seedlings were grown for 2 weeks under short days on medium containing the indicated concentrations of mitomycin C.

hormone. However, the ability of 2,4-D to promote root hair elongation (Pitts et al., 1988) was unaffected by the *rpn10-1* mutation, indicating that not all auxin responses were altered. Although untreated root hairs were shorter in *rpn10-1*, 2,4-D still was able to stimulate their elongation to an extent comparable to that in the wild type (Figure 6B).

Rpn10-1 Is Hypersensitive to ABA

In addition to cytokinins and auxins, the ubiquitin/26S proteasome pathway regulates responses to a variety of other plant hormones (Callis and Vierstra, 2000; Hellmann and Estelle, 2002). Thus, it was conceivable that other hormone signaling pathways were affected in *rpn10-1*, given its overall increase in ubiquitinated proteins (Figure 1D). Especially relevant was ABA, based on its role in suppressing seed germination (Finkelstein et al., 2002) and the poor germination of *rpn10-1* seeds (Figure 3A).

Using effects on overall seedling growth and root elongation as the assays, we found that *rpn10-1* plants responded normally to 22(*S*),23(*S*)-homobrassinolide, gibberellic acid 3, methyl jasmonate, salicylic acid, and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (J. Smalle, unpublished data). By contrast, *rpn10-1* seedlings exhibited a strong hypersensitivity to ABA. When germinated for 1 day in the absence of ABA and then transferred to medium containing increasing amounts of ABA, wild-type seedlings showed an increasing inhibition of leaf emergence and expansion, with higher concentrations (4 μM) also blocking expansion and greening of the cotyledons (Figure 7A) (Lopez-Molina et al., 2001). Relative to other Arabidopsis ecotypes, such as Columbia, Landsberg *erecta*, and Wassilewskija, we noted that C24 wild type appeared to be more sensitive to ABA by this assay. When *rpn10-1* seedlings were tested similarly, an ~10-fold increase in ABA sensitivity was observed, with as little as 0.4 μM ABA needed to arrest cotyledon development completely (Figure 7A). Even after weeks of incubation on ≥1 μM ABA, the *rpn10-1* seedlings remained arrested at this early developmental stage. A similar increase in ABA sensitivity was not seen for *rpn12a-1* (Figure 7B), suggesting that this effect on postgerminative development specifically requires RPN10. ABA also inhibited root elongation, with our results indicating that *rpn10-1* roots are hypersensitive. For seedlings grown for 4 days without ABA and then transferred to medium containing 1 μM ABA, the growth rate of *rpn10-1* roots was reduced by 50% compared with that of wild-type roots (Figure 7C). By comparison, the *rpn12a-1* mutant showed normal ABA sensitivity by this root assay (Smalle et al., 2002).

ABA also is important for regulating plant growth responses to osmotic stress, especially early in development, suggesting that it plays a role in preventing premature germination and growth under unfavorable conditions (Finkelstein et al., 2002; Rolland et al., 2002). In agreement, we found that *rpn10-1* seedlings were hypersensitive to NaCl and sucrose stress. Concentrations of NaCl (100 mM) or sucrose (5%) that did not visibly deter the early development of the wild type effectively inhibited the greening and expansion of *rpn10-1* (Figure 7D). *rpn10* mutants from other eukaryotes are hypersensitive to amino acid analogs (Girod et al., 1999; Li and Wang, 2002; Wojcik and DeMartino, 2002). Similarly, we found that *rpn10-1* has increased sensitivity to the Arg analog canavanine by the root elongation assay (J. Smalle, unpublished data).

Several reports have suggested that ABA arrests plant growth in part by directly influencing the cell division machinery (Finkelstein et al., 2002). Given the constitutive downregulation of *CDKA;1* in *rpn10-1* seedlings (Figures 5E to 5G), we tested

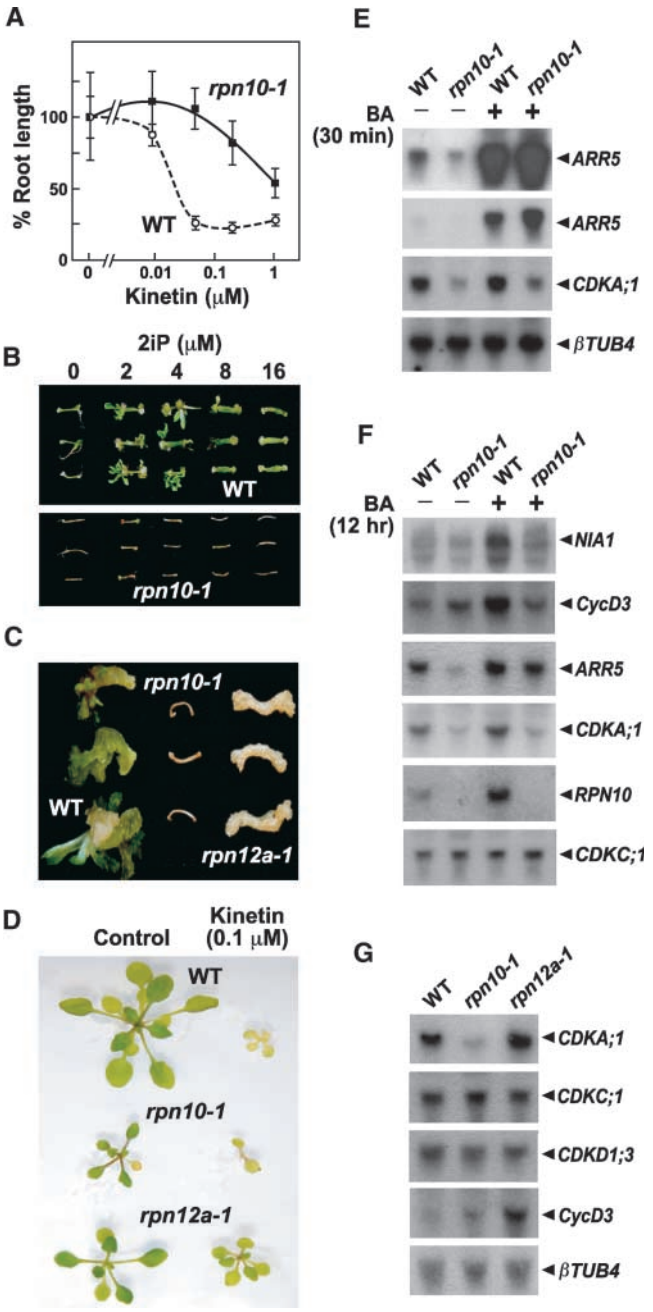

Figure 5. Cytokinin Responses and the Expression of Cytokinin-Regulated Genes Are Altered in the *rpn10-1* Mutant.

(A) Effect of a range of kinetin concentrations on root elongation in wild-type (WT) and homozygous *rpn10-1* seedlings. Seedlings were germinated and grown for 4 days in the absence of kinetin and transferred to medium containing the indicated kinetin concentrations. After an additional 9 days of growth, the mean root lengths of 10 plants were determined and are expressed as percentages of the values from the respective untreated controls (±SD).

(B) and **(C)** The induction of greening and shoot formation by 2iP is blocked in the *rpn10-1* mutant. Hypocotyl segments of 5-day-old wild-type, *rpn10-1*, and *rpn12a-1* seedlings were incubated for 2 weeks in the presence of 1 μM IAA supplemented with a range of 2iP concentrations **(B)** or for 3 weeks in the presence of 1 μM IAA supplemented with 4 μM 2iP **(C)**.

(D) Effect of kinetin on rosette leaf expansion. Wild-type, *rpn10-1*, and *rpn12a-1* seeds were germinated directly on 0.1 μM kinetin and grown for 3 weeks.

(E) mRNA levels from the *CDKA;1* gene and the cytokinin early-response gene *ARR5* are altered by the *rpn10-1* mutation. Wild-type and *rpn10-1* seedlings were grown in liquid medium for 10 days and then treated with BA for 30 min. Two autoradiographic exposures for *ARR5* are included to show the decreased expression of *ARR5* in untreated *rpn10-1* seedlings and the increased *ARR5* expression in treated *rpn10-1* seedlings.

(F) Cytokinin induction of the late-response genes *NIA1* and *CYCD3* is blocked in *rpn10-1*. Experimental conditions were the same as in **(E)** except that the seedlings were treated with BA for 12 h.

(G) mRNA levels for various cell cycle proteins in wild-type, *rpn10-1*, and *rpn12a-1* seedlings. Total RNA from seedlings grown in liquid medium for 10 days was subjected to RNA gel blot analysis with probes for the *CDK* genes *CDKA;1*, *CDKC;1*, and *CDKD1;3* and the cyclin gene *CYCD3*. Equal amounts of total RNA were confirmed by staining for rRNA with methylene blue (data not shown) and by reprobing the blots with *βTUB4* or *CDKC;1*.

by RNA gel blot analysis whether the expression of this *CDK* gene is regulated by ABA. Wild-type seedlings showed a selective reduction of *CDKA;1* mRNA (compared with *CDKC;1* and *RPN10* mRNA) when treated with 10 μM ABA for 6 h (Figure 7E). In *rpn10-1* seedlings, this level was reduced substantially even in the absence of ABA, possibly reflecting a constitutive repression of the *CDKA;1* gene. It has been reported that high concentrations of ABA (50 μM) transcriptionally upregulate the CDK inhibitor *ICK1/KRP1*, suggesting that increased levels of this protein could mediate some of the growth inhibition by ABA (Wang et al., 1998). However, *ICK1/KRP1* mRNA levels were not altered in *rpn10-1* seedlings, nor did treatment with an ABA concentration (10 μM) sufficient to downregulate *CDKA;1* change these levels (Figure 7E). As a result, we concluded that this CDK inhibitor is not involved in the growth rate reduction and ABA hypersensitivity of *rpn10-1*. Interestingly, the level of *ARR5* mRNA was reduced by ABA in both wild-type and mutant plants (Figure 7E). As a consequence, it is more likely that the reduction in *ARR5* mRNA in *rpn10-1* seedlings seen in Figure 5F was caused by increased sensitivity to ABA and not by decreased sensitivity to cytokinins.

Complementation of *rpn10-1*

To confirm that the *rpn10-1* phenotype was caused by alteration of the *RPN10* gene, we attempted to complement the mutation by ectopic expression of the wild-type cDNA under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV). When a number of independently transformed *rpn10-1 35S-RPN10* seedlings were examined, all of the aberrant traits associated with the *rpn10-1* mutation were restored to those of the wild type. These include the reduced growth rate and sta-

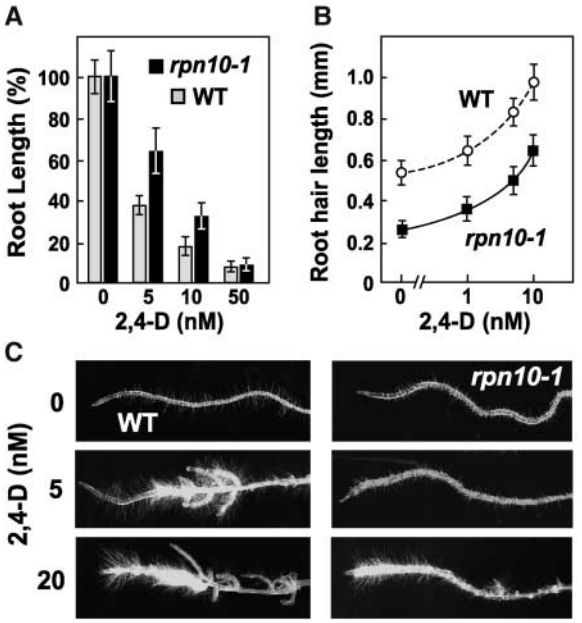

Figure 6. Roots of the *rpn10-1* Mutant Are Less Sensitive to the Auxin 2,4-D.

(A) Effect of the *rpn10-1* mutation on the inhibition of root elongation by 2,4-D. After 7 days on 2,4-D, the mean root lengths of 10 wild-type (WT) and *rpn10-1* plants were determined and are expressed as percentages of the lengths of the respective untreated controls (±SD).

(B) Effect of the *rpn10-1* mutation on lateral root formation after 10 days of treatment with various concentrations of 2,4-D.

(C) Effect of the *rpn10-1* mutation on 2,4-D–stimulated root hair elongation. Wild type (open circles) and *rpn10-1* (closed squares) seeds were germinated on medium containing 2,4-D, and the mean lengths of 40 root hairs were determined (±SD) after 7 days of growth.

men number, hypersensitivity to UV-B light and mitomycin C, decreased sensitivity to cytokinins, the inability to regenerate callus and shoots on IAA- and cytokinin-containing media, and hypersensitivity to ABA, NaCl, and sucrose (Figures 8A to 8C) (J. Smalle, unpublished data). We also observed no new phenotypes among the complementation lines, suggesting that potential *RPN10* misexpression caused by the use of the CaMV 35S promoter does not interfere with 26S proteasome assembly or activity. Introducing the *35S-RPN10* transgene in *rpn10-1* also decreased the levels of ubiquitinated proteins to wild-type levels (Figure 8D). Interestingly, the appearance of wild-type RPN10 reduced the levels of the RPN10-NPTII fusion protein in the *rpn10-1* background (Figure 8D). This loss suggests that the wild-type RPN10 protein effectively blocks the incorporation of the RPN10-NPTII fusion protein into the RP; the unincorporated fusion protein then is removed proteolytically.

rpn10-1 Selectively Stabilizes the ABA Response Transcription Factor ABI5

ABI5 is a member of a family of basic domain/Leu zipper transcription factors that helps confer ABA responsiveness in Arabidopsis by acting as both an activator and a repressor of gene expression (Finkelstein et al., 2002). Of interest are the observations that transgenic plants that constitutively overexpress *ABI5* have an ABA response phenotype similar to *rpn10-1* (arrested postgerminative development [Lopez-Molina et al., 2001]), whereas *abi5* null mutants respond in an opposite manner from *rpn10-1* (they are insensitive to ABA [Finkelstein and Lynch, 2000]). Lopez-Molina et al. (2001) previously showed that ABA can increase the levels of ABI5 through both transcriptional induction of the *ABI5* gene and increased accumulation of the protein, possibly by a mechanism that blocks its degradation by the 26S proteasome. Consequently, it is plausible that the ABA hypersensitivity of *rpn10-1* is mediated wholly or in part by the upregulation of *ABI5* expression and/or the selective stabilization of the ABI5 protein.

To test these possibilities, we compared the levels of the *ABI5* mRNA and protein in wild-type and *rpn10-1* seedlings before and after treatment with ABA. The *ABI5* mRNA was expressed at nearly undetectable levels in young Arabidopsis seedlings. As shown in Figure 7E, these levels were not increased significantly by the *rpn10-1* mutation or by treatment of wild-type and mutant seedlings with ABA. By contrast, the steady state level of the ABI5 protein was increased dramatically by the mutation, even in the absence of exogenous ABA (Figure 9A). Accumulation was highest in younger seedlings, in accord with previous reports showing that ABI5 synthesis is restricted to a short interval during early seedling development (Lopez-Molina et al., 2001). Five-day-old *rpn10-1* seedlings had the greatest enrichment of ABI5; its accumulation then decreased such that the level of ABI5 in 9-day-old *rpn10-1* seedlings was just slightly more than that in the wild type (Figures 9A and 9B). A doublet of ABI5 protein was detected (Figure 9A); the lower apparent molecular mass species was consistent with the size of the unmodified protein, with the higher, more diffuse species potentially representing a phosphorylated form (Lopez-Molina et al., 2001). In the presence of ABA, levels of ABI5 increased even further in *rpn10-1* plants and could be elicited by a concentration of ABA (0.2 μM) too low to affect ABI5 levels in wild-type plants (Figure 9B). Notably, the same low level of ABA also was sufficient to inhibit the postgerminative development of *rpn10-1* but not wild-type seedlings (Figure 7A), implicating the stabilization of ABI5 in the ABA-hypersensitive phenotype.

Analysis of two other ubiquitin/26S proteasome pathway substrates demonstrated that the increased amount of ABI5 in *rpn10-1* was caused by a selective stabilization of ABI5 and not by a general stabilization of all pathway substrates. Both phytochrome A (phyA) and long hypocotyl-5 (HY5) are regulators of Arabidopsis photomorphogenesis but are degraded by two different mechanisms within the pathway (Clough et al., 1999; Osterlund et al., 2000). Whereas phyA is stable in dark-grown plants but is degraded rapidly once plants are exposed to light, HY5 is degraded rapidly in dark-grown plants but stabilized once plants are exposed to light. As shown in Figures 9C and 9D, both phyA and HY5 were degraded normally in the *rpn10-1* background. phyA was removed rapidly during red light irradiation of dark-grown *rpn10-1* seedlings, with a half-life (~1 h) indistinguishable from that of the wild type. Likewise,

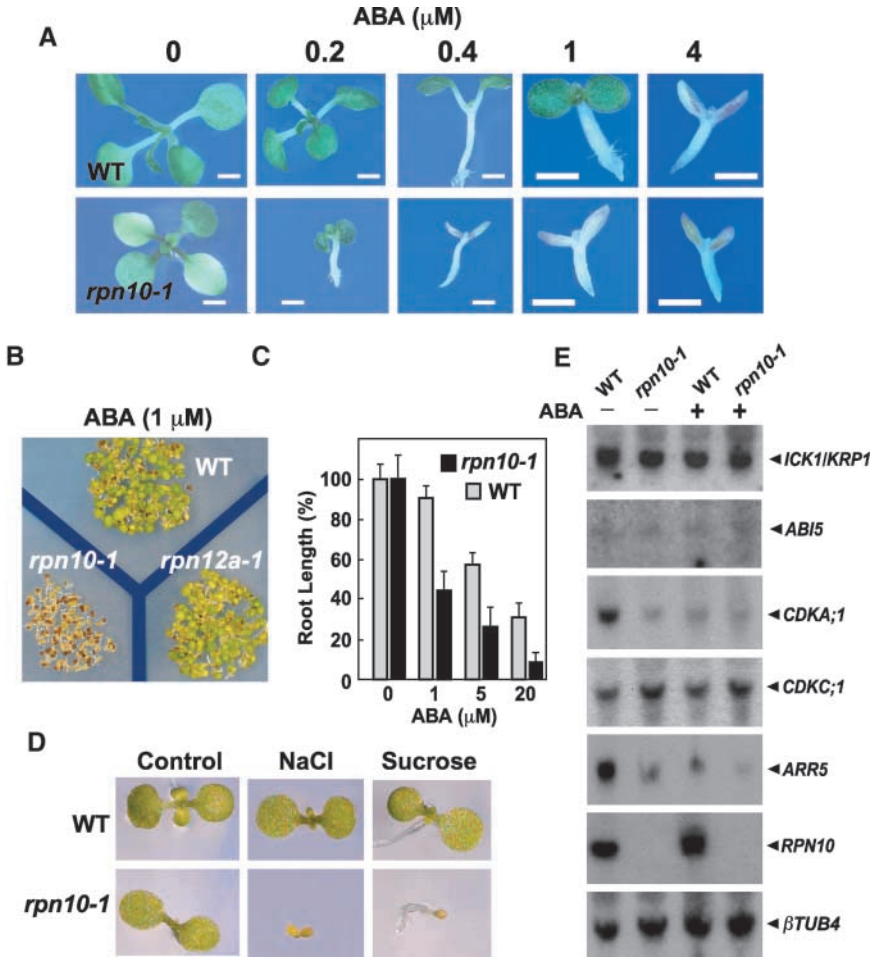

Figure 7. The *rpn10-1* Mutant Is Hypersensitive to ABA, NaCl, and Sucrose.

(A) Effect of a range of ABA concentrations on the expansion and greening of cotyledons and true leaves. Wild-type (WT) and *rpn10-1* seeds were stratified for 4 days at 4°C. After a 1-day incubation at 22°C, the seeds were transferred to medium containing the indicated concentrations of ABA and grown for 2 weeks under constant irradiation. Bars = 1 mm.

(B) Comparison of the effects of 1 μM ABA on *rpn10-1* and *rpn12a-1* seedlings. Conditions are the same as those described for **(A)**.

(C) Effect of ABA on root elongation. Wild-type and *rpn10-1* seeds were germinated without ABA; after 4 days, seedlings were transferred to medium containing the indicated ABA concentrations. After an additional 10 days of growth, the mean root lengths of 10 plants were determined and are expressed as percentages of the values of the respective untreated controls (±SD).

(D) Effects of NaCl and sucrose on the expansion and greening of *rpn10-1* cotyledons. Seeds of the wild type and *rpn10-1* were germinated and grown for 7 days on medium containing 100 mM NaCl or 5% sucrose.

(E) RNA gel blot analysis of gene expression in response to ABA. Wild-type and *rpn10-1* seedlings were grown in liquid medium for 7 days and then treated with 10 μM ABA for 6 h. Total RNA was subjected to RNA gel blot analysis with probes for *ABI5*, *ICK1/KRP1*, the *CDK* genes *CDKA;1* and *CDKC;1*, *ARR5*, and *RPN10*. Equal loads of total RNA were confirmed by staining for rRNA with methylene blue (data not shown) and by reprobing the blots with *βTUB4*.

HY5 did not accumulate in dark-grown *rpn10-1* and wild-type seedlings but did accumulate in both when the seedlings were exposed to light. The normal turnover rates of phyA and HY5 are consistent with the absence of photomorphogenic defects for the *rpn10-1* mutant (Figures 3A and 3D) expected if either or both proteins were stabilized (Clough et al., 1999; Osterlund et al., 2000).

DISCUSSION

Previous studies have implicated the ubiquitin/26S proteasome pathway in the perception of various environmental signals and hormones (Callis and Vierstra, 2000; Hellmann and Estelle, 2002). Here, we provide additional support for its role in ABA responses through the analysis of an exon trap mutant of

RPN10, a 26S proteasome subunit within the RP Base subcomplex. The *rpn10-1* allele synthesized a chimeric protein containing the N-terminal half of RPN10 appended to the sequence of NPTII. This truncation preserved the vWA domain identified as being important for tethering the Lid to the Base (at least in vitro) but was missing the C-terminal domain containing the UIM that binds polyubiquitin chains (Fu et al., 1998b, 2001). Consistent with this protein organization, the RPN10-NPTII fusion protein retained its ability to associate with the Arabidopsis 26S proteasome. However, this association was diminished greatly in the presence of wild-type RPN10, indicating that the fusion protein does not effectively compete with the native protein for binding to the complex. We obtained a similar result for an analogous NPTII fusion with RPN12a (Smalle et al., 2002), suggesting that appendages to various RP polypeptides can be useful in creating weak alleles for essential subunits.

Analysis of the resulting 26S proteasomes demonstrated that *rpn10-1* does not appreciably alter the structure, core subunit composition, and peptidase activity of the Arabidopsis holoenzyme. This finding implies that the observed phenotypes associated with *rpn10-1* most likely are caused by a specific loss of RPN10 activit(ies) rather than by more general defects in RP structure, activity, or levels. As such, the analysis of *rpn10-1* should provide insights into the specific function(s) of the RPN10 protein.

Two mutually compatible mechanisms may explain biochemically the phenotypic defects observed here for *rpn10-1* plants. The most obvious is that substitution of the C-terminal domain of RPN10 for NPTII removes or interferes with a critical activity in RPN10. In particular, the absence of the UIM may reduce the ability of the RP to deliver polyubiquitinated cargo to the 26S proteasome. Although the UIM does not appear to be critical in yeast RPN10 (Fu et al., 1998b), complementation of a *Physcomitrella rpn10Δ* strain with UIM mutants suggests that this domain plays a more important role in higher order eukaryotes (Girod et al., 1999). The second possibility relates to the decreased abundance of the fusion protein compared with the wild-type protein. Among the RPN subunits, RPN10 is unusual because a large fraction in crude extracts exists in an apparently free form not associated with the 26S complex (Haracska and Udvardy, 1995; van Nocker et al., 1996a). If this free RPN10 pool exist in vivo, one conceivable function is that it helps shuttle targets to the 26S proteasome by reversible binding. Lower amounts of the RPN10-NPTII fusion protein may affect this free pool and thus its shuttle function preferentially. It also is possible that the lower level of RPN10 concomitantly reduces the

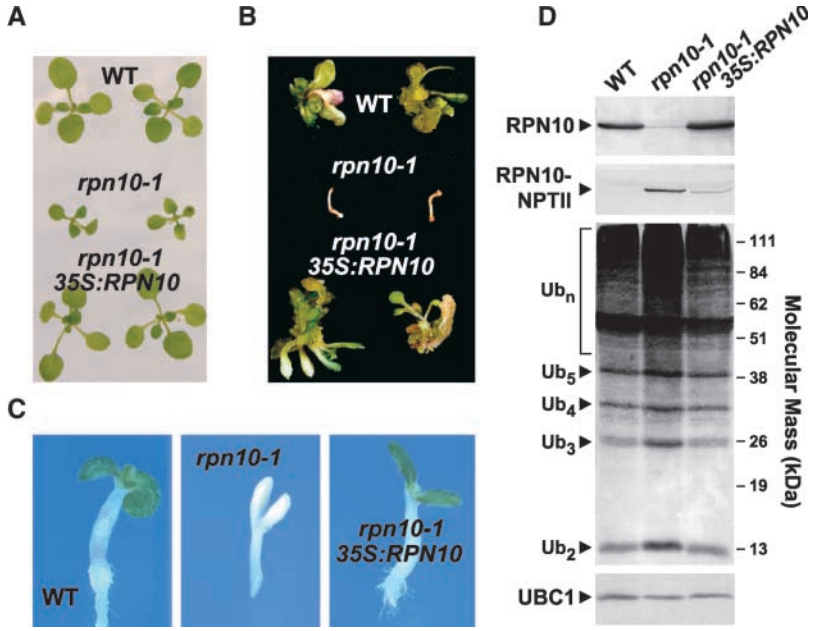

Figure 8. Complementation of the *rpn10-1* Mutation with *RPN10*.

Lines tested include the wild type (WT), *rpn10-1*, and *rpn10-1* transformed with a cDNA containing the *RPN10* coding region expressed under the control of the CaMV 35S promoter (*rpn10-1 35S-RPN10*).

(A) Growth for 2 weeks under a short-day photoperiod.

(B) Hormone-induced cell division in hypocotyl segments. Hypocotyl segments of 5-day-old seedlings were incubated for 3 weeks in the presence of 1 μM IAA and 4 μM 2iP.

(C) Effect of ABA on the expansion and greening of cotyledons. Seeds were stratified for 4 days at 4°C. After a 1-day incubation at 22°C, the seeds were transferred to medium containing 0.8 μM ABA and grown for 14 days.

(D) Levels of RPN10, RPN10-NPTII fusion protein, and polyubiquitinated proteins. Crude protein extracts were prepared from 7-day-old seedlings and subjected to SDS-PAGE and immunoblot analysis with antibodies against RPN10, NPTII, and ubiquitin (Ub). Analysis with anti-UBC1 antibodies was used to confirm equal protein loads.

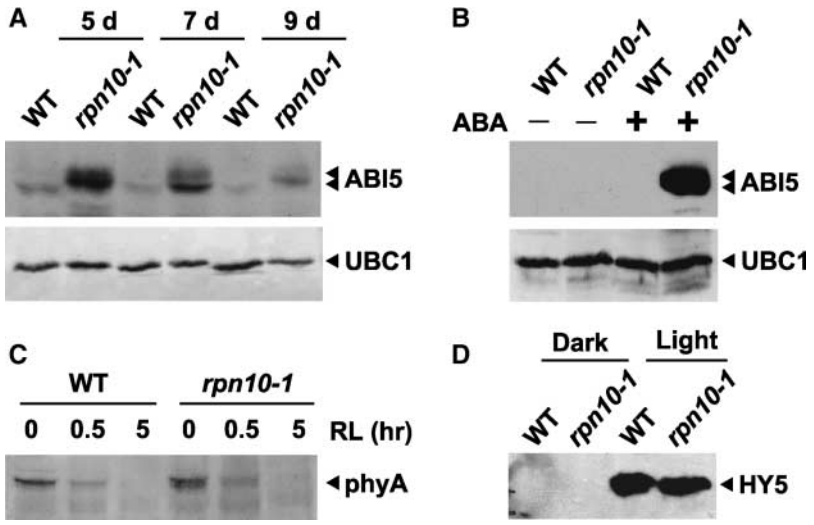

Figure 9. Selective Stabilization of the ABA Signaling Protein ABI5 in *rpn10-1* Seedlings.

(A) Immunoblot detection of ABI5 at various times after seed germination. Total protein was extracted from wild-type (WT) and *rpn10-1* seedlings of the indicated ages and subjected to SDS-PAGE and immunoblot analysis with anti-ABI5 antibodies.

(B) Immunoblot detection of ABI5 in response to ABA treatment. Seeds were transferred 1 day after stratification to medium containing 0.2 μM ABA and incubated for another 8 days. Immunoblot analysis was performed as described for **(A)**, except that a shorter exposure time was required to visualize the ABA-induced accumulation of ABI5 in *rpn10-1*. Analysis with anti-UBC1 antibodies was used to confirm equal protein loads.

(C) Turnover of phyA in wild-type and *rpn10-1* seedlings grown for 6 days in the dark and then exposed to continuous red light (RL). Immunoblots were probed with an anti-phyA monoclonal antibody.

(D) Levels of HY5 in wild-type and *rpn10-1* seedlings grown for 6 days in the dark followed by a 15-h exposure to white light. Immunoblots were probed with anti-HY5 antibodies.

level of the 26S proteasome by limiting the supply of this subunit. However, we found that the levels of 26S proteasome actually increased slightly in the *rpn10-1* background, likely attributable to a coordinated increase in the synthesis of the other subunits (Wojcik and DeMartino, 2002; P. Yang and J. Smalle, unpublished data). Moreover, it is unlikely that a dramatic reduction in 26S proteasome levels would generate the specific defects observed here.

The pleiotropic phenotype of *rpn10-1* plants indicates that the mutation affects a number of responses in Arabidopsis, a substantial portion of which can be explained by a flaw in ABA signaling. ABA plays numerous roles, including preventing precocious germination of seeds, arresting postgerminative development, delaying chloroplast development, inhibiting cell division, controlling pollen development, and protecting plants from salt and sucrose stress (Lopez-Molina et al., 2001; Finkelstein et al., 2002; Rolland et al., 2002). Importantly, defects in all of these processes were observed with the *rpn10-1* mutant and are consistent, in most cases, with an increase in ABA sensitivity. The reduced germination of *rpn10-1* seeds, especially soon after maturation, agrees with a role of ABA in preventing germination too early after seed drop or under unfavorable conditions (Finkelstein et al., 2002). For example, the maize *viviparous1* mutant, with accentuated precocious germination, affects a key component in ABA signaling (Suzuki et al., 2001). The enhanced chlorosis of *rpn10-1* plants is consistent with a stimulatory role of ABA in the senescence processes (Finkelstein et al., 2002).

Although the decreased cytokinin sensitivity of *rpn10-1* superficially links RPN10 to cytokinin signaling, this insensitivity is atypical and may be explained more easily by a change in ABA responsiveness. For example, *rpn10-1* seedlings are phenotypically less sensitive than *rpn12a-1* seedlings to cytokinin based on the ability of the hormone to stimulate cell division. However, rosette growth of *rpn10-1* is more sensitive to cytokinin than that of *rpn12a-1*. These differences also were seen at the molecular level, where *rpn12a-1* constitutively upregulated the late-cytokinin-induced genes *NIA1* and *CYCD3* but *rpn10-1* failed to induce both genes in the presence or absence of cytokinins (Figures 5E and 5F) (Smalle et al., 2002). In addition, mRNA levels for the cytokinin early-response gene *ARR5* were unaffected in *rpn12a-1* (Smalle et al., 2002) but were downregulated in *rpn10-1* in the absence of cytokinin (Figure 7E). The ARR5 protein was described initially as a repressor of both cytokinin responses and *ARR5* expression and appears to function as a feedback inhibitor to suppress cytokinin responses after the initial burst in cytokinin signaling (Hwang and Sheen, 2001). However, we show here that ABA, consistent with its ability to antagonize many cytokinin responses, also can decrease *ARR5* expression and that this repression is accentuated by the *rpn10-1* mutation. Thus, it is conceivable that altered *ARR5* expression in *rpn10-1* actually is caused by the ABA hypersensitivity. Furthermore, the observations that ABA inhibits cell division and *CYCD3* expression (Murray et al., 2001) also are consistent with the attenuated cytokinin-induced

cell division and the absence of *CYCD3* induction shown here for *rpn10-1* plants.

Changes in ABA and not cytokinin signaling also may be responsible for the repression of *rpn10-1* growth. In Arabidopsis, as in yeast, the levels and/or activities of CDKs appear to be important determinants of cell division and thus growth. For example, plant growth rates have been correlated with the levels of CDKs, in particular the CDKA subfamily (Granier et al., 2000; Beemster et al., 2002). Inhibiting CDKA activity subsequently inhibits growth by attenuating meristem activity (Wang et al., 1998; De Veylder et al., 2001). Interestingly, the *CDKA;1* gene has been reported to contain a putative ABA response element in its promoter region, suggesting that its expression is under the control of ABA (Chung and Parish, 1995). Hemerly et al. (1993) found that ABA can suppress *CDKA;1* expression using promoter–β-glucuronidase fusions in Arabidopsis protoplasts and roots. In agreement, we found here that ABA can suppress the accumulation of the natural *CDKA;1* mRNA and that this accumulation is suppressed constitutively in the *rpn10-1* mutant. Together, these findings suggest that ABA exerts its action on cell division by suppressing *CDKA;1* expression. Heightened sensitivity to ABA could exacerbate this suppression and thus negatively regulate growth even in the presence of cytokinins.

Heightened ABA responsiveness also may play a role in the hypersensitivity of *rpn10-1* plants to DNA-damaging agents. For example, the Arabidopsis *uvs66* mutant, identified first as a UV light–hypersensitive mutant, was found subsequently to have increased sensitivity to ABA (Albinsky et al., 1999). The DNA repair process may be connected to RPN10 via its potential association with RAD23 (Chen and Madura, 2002). Similar to the *rpn10-1* defect in Arabidopsis, the loss of *RAD23* function in yeast leads to UV light and mitomycin C hypersensitivity and the accumulation of ubiquitinated proteins (Lambertson et al., 1999; Chen and Madura, 2002). The Arabidopsis genome encodes four putative *RAD23* homologs, at least two of which can complement yeast *rad23Δ* mutants (Sturm and Lienhard, 1998). Their connection to ABA and DNA damage in plants is not yet known. However, a rice homolog of RAD23 has been found to interact by yeast two-hybrid assay with VP1, another key signaling protein in the ABA response pathway (Schultz and Quatrano, 1997). The ortholog of VP1 in Arabidopsis is ABI3, a transcription factor that binds ABI5 to regulate the expression of ABA response genes (Gampala et al., 2002). Therefore, it is conceivable that RPN10, RAD23, and ABI3 work in concert to adjust ABI5 levels and thus regulate ABA-induced responses to signals such as DNA damage.

Finally, increased ABA sensitivity also may explain the small decrease in the auxin sensitivity of *rpn10-1* roots. Even though auxin signaling clearly involves the ubiquitin/26S proteasome pathway through the TIR1-mediated degradation of AUX/IAA proteins (Hellmann and Estelle, 2002), it is possible that the decreased auxin sensitivity of *rpn10-1* roots is related to the increased levels of ABA-signaling proteins such as ABI5. In support of this notion, Suzuki et al. (2001) recently showed that overexpression of the rice ABI3 homolog VP1 in Arabidopsis generated plants in which auxin-induced lateral root formation was inhibited by ABA.

Assuming that many of the phenotypes of *rpn10-1* are induced by a heightened sensitivity to ABA, we considered it likely that RPN10 plays a role in the degradation of one or more activators/repressors of ABA action in Arabidopsis. Previous studies have suggested that the ABA response regulator ABI5 is a target of the 26S proteasome by showing that the CP inhibitor MG132 increased the level of the protein dramatically (Lopez-Molina et al., 2001). Here, we confirmed this notion by demonstrating that the level of ABI5 was increased specifically by the *rpn10-1* mutation. By contrast, two other targets of the Arabidopsis ubiquitin/26S proteasome pathway, phyA and HY5, were degraded normally. Intriguingly, ABA treatment of the *rpn10-1* seedlings further enhanced ABI5 levels, suggesting that ABA can increase ABI5 half-life directly. Given the fact that many phenotypes of *rpn10-1* plants can be explained by ABI5 protein regulation (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001), it is possible that ABI5 is one of the prime targets of RPN10. However, we consider it unlikely that ABI5 is the sole target, because of the general increase in ubiquitinated proteins in the *rpn10-1* background. Additional targets could include other components of the ABA-signaling cascade, such as ABI3 and other members of the ABI5 family (Gampala et al., 2002; Kim et al., 2002).

One revealing property of ABI5 is that its action requires ABA. For example, plants that overexpress *ABI5* behave like wild-type plants in the absence of exogenous ABA and only elicit their characteristic phenotypes upon treatment with the hormone (Lopez-Molina et al., 2001). This finding suggests that the enhanced synthesis of ABI5 protein alone is insufficient for its function but must be combined with repressed degradation, presumably to allow the accumulation of an ABA-activated form. Such a two-part mechanism could explain the differences in developmental phenotypes of *ABI5*-overexpressing plants with a mutant such as *rpn10-1* that stabilizes the protein. The *rpn10-1* phenotype also suggests a more general role for ABI5 in Arabidopsis development, as opposed to the very limited function in postgerminative development ascribed to it previously (Lopez-Molina et al., 2001). In fact, several studies have documented ABI5 expression throughout plant development, in agreement with the highly pleiotropic phenotype of *rpn10-1* plants (Signora et al., 2001; Brocard et al., 2002).

How ABI5 is degraded selectively is not yet known. The kinetics of ABI5 accumulation with and without ABA suggest that ABI5 normally is short-lived. Although other mechanisms are possible, the simplest is that ABI5 turnover is achieved through the interaction of ABI5 with RPN10. This interaction could be direct or indirect, could be dependent or independent of prior ubiquitination, and/or could exploit adaptor proteins such as RAD23 or a RAD23/ABI3 complex. ABA could stabilize ABI5 by preventing this association. One possible mechanism for the ABA-induced stabilization of ABI5 is via phosphorylation of the protein (Lopez-Molina et al., 2001). As a consequence, ABI5 may be related to a number of other regulatory proteins that exploit phosphorylation to control their turnover by the ubiquitin/26S proteasome pathway (Hershko and Ciechanover, 1998). Although phosphorylation most often stimulates ubiquitination and 26S proteasome–dependent degradation, ABI5 may behave

like the mammalian c-MOS mitogen-activated protein kinase kinase kinase, in which phosphorylation promotes its stabilization (Sheng et al., 2002).

It has been assumed previously that the selectivity of the ubiquitin/26S proteasome pathway is achieved largely by the ubiquitination steps (Hershko and Ciechanover, 1998; Gagne et al., 2002). The 26S proteasome was expected to provide little specificity, using the same route to degrade its varied substrates. However, recent genetic analyses of the yeast complex have shown that the loss of individual RP subunits can lead to the selective stabilization of specific targets, demonstrating that the 26S proteasome also can distinguish among its substrates and presumably use different routes for entry (van Nocker et al., 1996b; Bailly and Reed, 1999). The combined analyses of Arabidopsis mutants of the RPN12a and RPN10 subunits are consistent with this notion. Each appears to affect plant growth and development in distinct ways, implying that different sets of targets are stabilized. The selective stabilization of ABI5 over phyA and HY5 in *rpn10-1* plants shows that this subunit has a particularly discriminating function in substrate selection. Such preference is apparent for its yeast ortholog, which also is involved in removing a limited subset of ubiquitin pathway targets (van Nocker et al., 1996b). The ability of RPN10 to promote the selective recruitment of substrates may be related to its ability to bind polyubiquitin chains and/or potentially help adaptor proteins such as RAD23 and DSK2 associate with the protease. Clearly, identifying the proteins that interact with RPN10 will be critical to defining how it helps deliver appropriate cargo to the 26S proteasome.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* C24 ecotype was used as wild type for all phenotypic assays of *rpn10-1*. Unless noted otherwise, plants were grown under sterile conditions on solid Gamborg's B5 growth medium (GM) (Gibco BRL, Gaithersburg, MD) containing 1% sucrose. Hormones were obtained from Sigma (St. Louis, MO). To reduce the poor germination of *rpn10-1* seeds, phenotypic assays were performed with seeds stored at 24°C for at least 1 month before sowing. For monitoring of root elongation, lateral root formation, and root hair elongation, a minimum of 10 seedlings were transferred at 4 days after germination on GM, and the plates were held in the vertical position for 7 days. For the shoot induction experiments, root and hypocotyl segments were dissected from 5-day-old light-grown seedlings, transferred to GM containing 1 μM indoleacetic acid with or without a range of 2-isopentyladenine concentrations, and incubated for another 2 weeks in continuous light. To test the effects of abscisic acid on postgermination development, seeds first were stratified in the dark at 4°C for 4 days on GM. The seeds then were placed under continuous irradiation at 22°C. After 1 day, the seeds were transferred to GM containing a range of abscisic acid concentrations and incubated for another 2 weeks. To test the response to NaCl and sucrose, seeds were sown directly on GM containing these compounds; after stratification, the plates were placed under continuous irradiation at 22°C for 1 week. For responses to UV-B light and mitomycin C, seedlings exposed to an 8-h-light/16-h-dark photoperiod (short days) were either placed on GM containing mitomycin C (Sigma) or irradiated every day for 14 days with 30-s or 3-min exposures to UV-B light provided by a UV Transilluminator 2000 (Bio-Rad Laboratories).

26S Proteasome Purification and Assay

The 26S proteasome was partially purified from 3-week-old seedlings grown in a 16-h-light/8-h-dark photoperiod. Plants were frozen to liquid nitrogen temperatures, pulverized, and ground in 1.25 volumes of buffer A (50 mM sodium phosphate, pH 8.0, 2 mM $MgCl_2$, 10% glycerol, 1 mM 2-mercaptoethanol, 1 mM ATP, and 5% polyvinylpyrrolidone 360). After filtering through cheesecloth, the extract was clarified for 15 min at 3,000*g* and twice more for 20 min at 35,000*g*. The supernatant was subjected to fast protein liquid chromatography using a 1- × 1-cm Resource Q column (Bio-Rad) equilibrated with buffer B (buffer A without 5% polyvinylpyrrolidone 360) (Fu et al., 2001). The proteins were eluted using a gradient of 0.1 to 0.5 M NaCl in buffer B at a flow rate of 0.3 mL/min. Fractions containing the 26S proteasome were identified by peptidase activity using the substrate *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Sigma) and by immunoblot analysis with appropriate antibodies (Smalle et al., 2002).

Immunoblot Analysis

Immunoblot analysis was performed according to Smalle et al. (2002). Antibodies against ABI5, HY5, RPN5, RPT1, PBF1, PAC1, RPN10 (previously designated MBP1), ubiquitin, and UBC1 were as described (van Nocker et al., 1996a; Osterlund et al., 2000; Lopez-Molina et al., 2001; Smalle et al., 2002). Anti-NPTII antibodies were obtained from U.S. Biological (Swampscott, MA). Arabidopsis phytochrome A was detected with the monoclonal antibody 073D generated against oat phytochrome A (Clough et al., 1999).

RNA Isolation, 5′ Rapid Amplification of cDNA Ends PCR, and RNA Gel Blot Analysis

Total RNA was isolated and subjected to gel blot analysis according to Smalle et al. (2002). 5′ rapid amplification of cDNA ends was conducted as described by Babiychuk et al. (1997). ^{32}P-labeled riboprobes were synthesized with T7 or SP6 RNA polymerase using the appropriate linearized plasmids and the Riboprobe Gemini II core system (Promega, Madison, WI). The *CYCD3*, *βTUB4*, *ARR5*, *NIA1*, and *CDKA;1* templates were as described (Smalle et al., 2002). The *RPN10* template used the pJSRPN10 plasmid linearized with SpeI-PstI. This plasmid was created by PCR amplification of the *RPN10* cDNA from a cDNA library using primers 5′-TATCGACACGATGGTTCTCGAGGCGACTAT-3′ and 5′-AATTCAGTTCCAATCTCTATCAAACCAAAG-3′ and insertion of the product into pGEM-T (Promega). The *NPTII* template used the pJSNPTII plasmid linearized with ApaI-NcoI. This plasmid was created by PCR amplification of the *NPTII* coding frame from the genomic DNA of the *rpn12a-1* mutant (Smalle et al., 2002) using primers 5′-TGCCCTGAATGAACTGCAGGACGAGGCAGC-3′ and 5′-TCAGAAGAACTCGTCAAGAAGGCGATA-3′ and insertion of the product into pGEM-T. The *ABI5* template used the pJSABI5 plasmid linearized with NotI-NcoI. This plasmid was created by PCR amplification of the *ABI5* coding region from Columbia genomic DNA using primers 5′-TCCATATTTCAAACTAGTTTGTGTGAATACAGA-3′ and 5′-GTTCTGTCTCCGACTAGTTCTCCATCTTCCTAT-3′ and insertion of the product into pGEM-T. The *CDKC;1*, *CDKD1;3*, and *ICK1/KRP1* templates used the EST clones 105H14, 122J8, and 156J24, respectively, linearized with KpnI-PstI. RNA gel blot signal intensities were estimated using the NIH Image program (National Institutes of Health, Bethesda, MD).

rpn10-1 Complementation

The *RPN10* cDNA was amplified by PCR using primers 5′-TCTCTCTGCTTATCGACACGATGGTTCTCG-3′ and 5′-AATTCAGTTCCAATC-

TAGATCAAACCAAAGAAACAA-3′ designed to contain an XbaI site at both ends. The resulting product was digested with XbaI and inserted downstream of the *Cauliflower mosaic virus* 35S promoter into the binary T-DNA vector pGSVE9, which was digested similarly (E. Babiychuk and S. Kushnir, unpublished data). The vector was introduced into *Agrobacterium tumefaciens* strain AtC58Rif^R(pMP90), which was used to transform homozygous *rpn10-1* seedlings by the floral-dip method. The presence of the *35S-RPN10* transgene and the *rpn10-1* mutation were followed by hygromycin and kanamycin resistance, respectively. T3 progeny resulting from self-crosses that were homozygous for both kanamycin and hygromycin resistance were analyzed for phenotypic complementation.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

ACKNOWLEDGMENTS

We thank Luis Lopez-Molina for providing the ABI5 antibodies, Xing Wang Deng for providing the HY5 antibodies, and the ABRC for providing the EST clones. This work was supported by grants from the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (00-35301-9040), the National Science Foundation Arabidopsis 2010 Program (MCB-0115870), and the Research Division of the University of Wisconsin College of Agriculture and Life Sciences (Hatch 142-E443) to R.D.V. and by a North Atlantic Treaty Organization research fellowship to J.S.

Received November 13, 2002; accepted January 24, 2003.

REFERENCES

Albinsky, D., Masson, J.E., Bogucki, A., Afsar, K., Vass, I., Nagy, F., and Paszkowski, J. (1999). Plant responses to genotoxic stress are linked to an ABA/salinity signaling pathway. Plant J. **17,** 73–82.

Babiychuk, E., Fuangthong, M., Van Montagu, M., Inze, D., and Kushnir, S. (1997). Efficient gene tagging in *Arabidopsis thaliana* using a gene trap approach. Proc. Natl. Acad. Sci. USA **94,** 12722–12727.

Bailly, E., and Reed, S.I. (1999). Functional characterization of Rpn3 uncovers a distinct 19S proteasomal subunit requirement for ubiquitin-dependent proteolysis of cell cycle regulatory proteins in budding yeast. Mol. Cell. Biol. **19,** 6872–6890.

Beemster, G.T., De Vusser, K., De Tavernier, E., De Bock, K., and Inze, D. (2002). Variation in growth rate between Arabidopsis ecotypes is correlated with cell division and A-type cyclin-dependent kinase activity. Plant Physiol. **129,** 854–864.

Brocard, I.M., Lynch, T.J., and Finkelstein, R.R. (2002). Regulation and role of the Arabidopsis *Abscisic Acid-Insensitive 5* gene in abscisic acid, sugar, and stress response. Plant Physiol. **129,** 1533–1543.

Callis, J., and Vierstra, R.D. (2000). Protein degradation in signaling. Curr. Opin. Plant Biol. **3,** 381–386.

Chen, L., and Madura, K. (2002). Rad23 promotes the targeting of proteolytic substrates to the proteasome. Mol. Cell. Biol. **22,** 4902–4913.

Chung, S.K., and Parish, R.W. (1995). Studies on the promoter of the *Arabidopsis thaliana CDC2a* gene. FEBS Lett. **362,** 215–219.

Clough, R.C., Jordan-Beebe, E.T., Lohman, K.N., Marita, J.M., Walker, J.M., Gatz, C., and Vierstra, R.D. (1999). Sequences within both the N- and C-terminal domains of phytochrome A are required for PFR ubiquitination and degradation. Plant J. **17,** 155–167.

D'Agostino, I.B., Deruere, J., and Kieber, J.J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol. **124,** 1706–1717.

De Veylder, L., Beeckman, T., Beemster, G.T., Krols, L., Terras, F., Landrieu, I., van der Schueren, E., Maes, S., Naudts, M., and Inze, D. (2001). Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell **13,** 1653–1668.

Elsasser, S., Gali, R.R., Schwickart, M., Larsen, C.N., Leggett, D.S., Muller, B., Feng, M.T., Tubing, F., Dittmar, G.A., and Finley, D. (2002). Proteasome subunit RPN1 binds ubiquitin-like protein domains. Nat. Cell. Biol. **4,** 725–730.

Finkelstein, R.R., Gampala, S.S., and Rock, C.D. (2002). Abscisic acid signaling in seeds and seedlings. Plant Cell **14** (suppl.), S15–S45.

Finkelstein, R.R., and Lynch, T.J. (2000). The Arabidopsis abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. Plant Cell **12,** 599–609.

Fu, H., Doelling, J.H., Arendt, C.S., Hochstrasser, M., and Vierstra, R.D. (1998a). Molecular organization of the 20S proteasome gene family from *Arabidopsis thaliana*. Genetics **149,** 667–692.

Fu, H., Doelling, J.H., and Vierstra, R.D. (1999a). Structural and functional analysis of the six regulatory particle triple-A ATPase subunits from the Arabidopsis 26S proteasome. Plant J. **18,** 529–539.

Fu, H., Girod, P.A., Doelling, J.H., van Nocker, S., Hochstrasser, M., Finley, D., and Vierstra, R.D. (1999b). Structure and functional analysis of the 26S proteasome subunits from plants. Mol. Biol. Rep. **26,** 137–146.

Fu, H., Reis, N., Lee, Y., Glickman, M., and Vierstra, R.D. (2001). Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. EMBO J. **20,** 7096–7107.

Fu, H., Sadis, S., Rubin, D.M., Glickman, M., van Nocker, S., Finley, D., and Vierstra, R.D. (1998b). Multiubiquitin chain binding and protein degradation are mediated by distinct domains within the 26S proteasome subunit Mcb1. J. Biol. Chem. **273,** 1970–1981.

Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M., and Vierstra, R.D. (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. Proc. Natl. Acad. Sci. USA **99,** 11519–11524.

Gampala, S.S., Finkelstein, R.R., Sun, S.S., and Rock, C.D. (2002). ABI5 interacts with abscisic acid signaling effectors in rice protoplasts. J. Biol. Chem. **277,** 1689–1694.

Girod, P.A., Fu, H., Zryd, J.P., and Vierstra, R.D. (1999). Multiubiquitin chain binding subunit MCB1 (RPN10) of the 26S proteasome is essential for developmental progression in *Physcomitrella patens*. Plant Cell **11,** 1457–1471.

Glickman, M.H. (2000). Getting in and out of the proteasome. Semin. Cell Dev. Biol. **11,** 149–158.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., and Finley, D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell **94,** 615–623.

Granier, C., Inze, D., and Tardieu, F. (2000). Spatial distribution of cell division rate can be deduced from that of p34(cdc2) kinase activity in maize leaves grown at contrasting temperatures and soil water conditions. Plant Physiol. **124,** 1393–1402.

Haracska, L., and Udvardy, A. (1995). Cloning and sequencing a non-ATPase subunit of the regulatory complex of the Drosophila 26S protease. Eur. J. Biochem. **231,** 720–725.

Hellmann, H., and Estelle, M. (2002). Plant development: Regulation by protein degradation. Science **297,** 793–797.

Hemerly, A.S., Ferreira, P., de Almeida Engler, J., Van Montagu, M., Engler, G., and Inze, D. (1993). *CDC2a* expression in Arabidopsis is linked with competence for cell division. Plant Cell **5,** 1711–1723.

Hensel, L.L., Grbic, V., Baumgarten, D.A., and Bleecker, A.B. (1993). Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in Arabidopsis. Plant Cell **5,** 553–564.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. **67,** 425–479.

Hofmann, K., and Falquet, L. (2001). A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. Trends Biochem. Sci. **26,** 347–350.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature **413,** 383–389.

Kim, S.Y., Ma, J., Perret, P., Li, Z., and Thomas, T.L. (2002). Arabidopsis ABI5 subfamily members have distinct DNA-binding and transcriptional activities. Plant Physiol. **130,** 688–697.

Kominami, K., et al. (1997). Yeast counterparts of subunits S5a and p58 (S3) of the human 26S proteasome are encoded by two multicopy suppressors of *nin1-1*. Mol. Biol. Cell **8,** 171–187.

Lam, Y.A., Lawson, T.G., Velayutham, M., Zweier, J.L., and Pickart, C.M. (2002). A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. Nature **416,** 763–767.

Lambertson, D., Chen, L., and Madura, K. (1999). Pleiotropic defects caused by loss of the proteasome-interacting factors Rad23 and Rpn10 of *Saccharomyces cerevisiae*. Genetics **153,** 69–79.

Li, Z., and Wang, C.C. (2002). Functional characterization of the eleven non-ATPase subunit proteins in the trypanosome 19 S proteasomal regulatory complex. J. Biol. Chem. **277,** 42686–42693.

Lopez-Molina, L., Mongrand, S., and Chua, N.H. (2001). A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc. Natl. Acad. Sci. USA **98,** 4782–4787.

Murray, J.A.H., Doonan, J., Riou-Khamlichi, C., Meijer, M., and Oakenfull, E.A. (2001). G1 cyclins, cytokinins and the regulation of the G1/S transition. In The Plant Cell Cycle and Its Interfaces, D. Frances, ed (Sheffield, UK: CRC Press), pp. 19–41.

Osterlund, M.T., Hardtke, C.S., Wei, N., and Deng, X.W. (2000). Targeted destabilization of HY5 during light-regulated development of Arabidopsis. Nature **405,** 462–466.

Piotrowski, J., Beal, R., Hoffman, L., Wilkinson, K.D., Cohen, R.E., and Pickart, C.M. (1997). Inhibition of the 26 S proteasome by polyubiquitin chains synthesized to have defined lengths. J. Biol. Chem. **272,** 23712–23721.

Pitts, R.J., Cernac, A., and Estelle, M. (1988). Auxin and ethylene promote root hair elongation in Arabidopsis. Plant J. **16,** 553–560.

Riou-Khamlichi, C., Huntley, R., Jacqmard, A., and Murray, J.A. (1999). Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science **283,** 1541–1544.

Rolland, F., Moore, B., and Sheen, J. (2002). Sugar sensing and signaling in plants. Plant Cell **14** (suppl.), S185–S205.

Saeki, Y., Saitoh, A., Toh-e, A., and Yokosawa, H. (2002). Ubiquitin-like proteins and Rpn10 play cooperative roles in ubiquitin-dependent proteolysis. Biochem. Biophys. Res. Commun. **293,** 986–992.

Schultz, T.F., and Quatrano, R.S. (1997). Characterization and expression of a rice *RAD23* gene. Plant Mol. Biol. **34,** 557–562.

Sheng, J., Kumagai, A., Dunphy, W.G., and Varshavsky, A. (2002). Dissection of the c-MOS degron. EMBO J. **21,** 6061–6071.

Shibahara, T., Kawasaki, H., and Hirano, H. (2002). Identification of the 19S regulatory particle subunits from the rice 26S proteasome. Eur. J. Biochem. **269,** 1474–1483.

Signora, L., De Smet, I., Foyer, C.H., and Zhang, H. (2001). ABA plays a central role in mediating the regulatory effects of nitrate on root branching in Arabidopsis. Plant J. **28,** 655–662.

Smalle, J., Kurepa, J., Yang, P., Babiychuk, E., Kushnir, S., Durski, A., and Vierstra, R.D. (2002). Cytokinin growth responses in Arabidopsis involve the 26S proteasome subunit RPN12. Plant Cell **14,** 17–32.

Sturm, A., and Lienhard, S. (1998). Two isoforms of plant RAD23 complement a UV-sensitive *rad23* mutant in yeast. Plant J. **13,** 815–821.

Suzuki, M., Kao, C.Y., Cocciolone, S., and McCarty, D.R. (2001). Maize VP1 complements Arabidopsis *abi3* and confers a novel ABA/auxin interaction in roots. Plant J. **28,** 409–418.

Takeuchi, J., Fujimuro, M., Yokosawa, H., Tanaka, K., and Toh-e, A. (1999). Rpn9 is required for efficient assembly of the yeast 26S proteasome. Mol. Cell. Biol. **19,** 6575–6584.

Vandepoele, K., Raes, J., De Veylder, L., Rouze, P., Rombauts, S., and Inze, D. (2002). Genome-wide analysis of core cell cycle genes in Arabidopsis. Plant Cell **14,** 903–916.

van Nocker, S., Deveraux, Q., Rechsteiner, M., and Vierstra, R.D. (1996a). Arabidopsis MBP1 gene encodes a conserved ubiquitin recognition component of the 26S proteasome. Proc. Natl. Acad. Sci. USA **93,** 856–860.

van Nocker, S., Sadis, S., Rubin, D.M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R.D. (1996b). The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. Mol. Cell. Biol. **16,** 6020–6028.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R., III, Koonin, E.V., and Deshaies, R.J. (2002). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science **298,** 611–615.

Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: A molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. **68,** 1015–1068.

Wang, H., Qi, Q., Schorr, P., Cutler, A.J., Crosby, W.L., and Fowke, L.C. (1998). ICK1, a cyclin-dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. Plant J. **15,** 501–510.

Wilkinson, C.R., Ferrell, K., Penney, M., Wallace, M., Dubiel, W., and Gordon, C. (2000). Analysis of a gene encoding Rpn10 of the fission yeast proteasome reveals that the polyubiquitin-binding site of this subunit is essential when Rpn12/Mts3 activity is compromised. J. Biol. Chem. **275,** 15182–15192.

Wojcik, C., and DeMartino, G.N. (2002). Analysis of Drosophila 26 S proteasome using RNA interference. J. Biol. Chem. **277,** 6188–6197.

Yu, X., Sukumaran, S., and Morton, L. (1998). Differential expression of the Arabidopsis *NIA1* and *NIA2* genes: Cytokinin-induced nitrate reductase activity is correlated with increased *NIA1* transcription and mRNA levels. Plant Physiol. **116,** 1091–1096.